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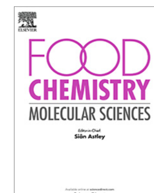

Beet (*Beta vulgaris* L.) stalk and leaf supplementation changes the glucose homeostasis and inflammatory markers in the liver of mice exposed to a high-fat diet

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ABSTRACT

Although beet stalks and leaves are not consumed and are usually discarded, they are an important source of bioactive flavonoids possessing antioxidant and anti-inflammatory activity. The aim of this study was to assess the effect of supplementation with beet stalks and leaves on metabolic parameters and glucose homeostasis in mice exposed to a high-fat diet. Six-week-old male Swiss mice were randomly divided into five experimental groups submitted to either standard diet (CT) or high-fat diet (HF), and HF-fed mice were subdivided into three treatment groups supplemented with oven-dehydrated beet stalks and leaves (SL), lyophilized beet stalks and leaves (Ly) or beet stalk and leaf extract (EX). Supplementation with SL promoted a mild improvement in the glucose homeostasis and decreased the protein levels of TNFα with no alterations in hepatic triglyceride content. It remains to be clarified if the enhancement in the glucose homeostasis observed in HFSL could be a consequence of improvement in pancreatic insulin secretion and/or glucose uptake from skeletal muscle and white adipose tissues.

1. Introduction

Obesity is associated with a high incidence of many metabolic disorders, including type 2 diabetes mellitus (DM2) and cardiovascular diseases. DM2 may be due to evolution of the glucose intolerance observed in metabolic syndrome, which is also associated with excessive weight gain and a sedentary lifestyle (World Health Organization, 2020). Hepatic disorders, such as non-alcoholic fatty liver disease (NAFLD), could be associated with obesity and have been considered by many authors the hepatic manifestation of metabolic syndrome (Rinella, 2015). One alternative to minimize the obesity associated diseases is to change the lifestyle with improvements in the usual diet quality (Bagherniya, Nobili, Blesso, & Sahebkar, 2018; Salomone et al., 2020).

It is well known that dietary pattern are one of the main causes associated with obesity and that consumption of fruits and vegetables provides health benefits. One example is beetroot (*Beta vulgaris* L.), which presents a high concentration of phenolic compounds and other substances, such as betalains, in its composition which have a positive effect on health (Vidal, López-Nicolás, Gandía-Herrero, & García-Carmona, 2014; Vulić et al., 2012; Vulić et al., 2014). Additionally, to the antioxidative properties and hepatoprotective activity (Vulić et al., 2014), some authors have observed that consumption of beetroot or its derivative products is able to improve sports performance, glycaemic control and blood pressure (Baião et al., 2019; Clifford, Howatson, West, & Stevenson, 2015; Coles & Clifton, 2012; Daab et al., 2020; Gilchrist et al., 2014; Jajja et al., 2014; Mirmiran, Houshialsadat, Gaeini, Bahadoran, & Azizi, 2020). However, despite the positive effects of beetroot or its derivatives on human health,

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there are few studies on non-conventional parts of beetroot such as stalks and leaves.

In some countries, beetroot stalks and leaves are considered food waste and are commonly discarded. Some fruits and vegetables yield between 25% and 30% of nonedible products (Ajila, Bhat, & Prasada Rao, 2007; Amaya-Cruz et al., 2015). However, different types of flavonoids (myricetin, quercetin, rutin and kaempferol) considered bioactive phytochemicals are found in beet leaves (Koubaier et al., 2014). Recent studies have shown different positive effects on metabolic parameters from treatment or supplementation with these phenolic compounds. Ferulic acid treatment to DM2 in rats, for example, improves insulin sensitivity and hepatic glycogenesis (Narasimhan, Chinnaiyan, & Karundevi, 2015). Kulabas et al. (2018) observed that *Lavandula stoechas* L. extract was effective in the treatment/prevention of insulin resistance and inflammation, attributed to the presence of caffeic acid, apigenin, luteolin, rosmarinic acid and its methyl ester, 4-hydroxybenzoic acid, vanillic acid, ferulic acid and salicylic acid. Other flavonoids, such as vitexin-2-O-rhamnoside and other vitexin derivatives, are also found in beet stalks and leaves. These compounds are able to attenuate the deleterious effects of a high-fat diet on oxidative damage in the liver in mice (Lorizola et al., 2018). According to Bumke-Vogt et al. (2014), administration of the flavones apigenin and luteolin to cells from human liver carcinomas has shown antidiabetic potential and reduces hepatic steatosis.

Thus, supplementation with beet (*Beta vulgaris* L.) stalks and leaves, or even extracts rich in the bioactive compounds, may improve the metabolic parameters involved in gluconeogenesis and liver function. So, our aim was to evaluate the effect of supplementation with beet (*Beta vulgaris* L.) stalks and leaves on glucose homeostasis and insulin resistance markers in the liver of mice exposed to a high-fat diet.

2. Materials and methods

Organic beetroot (*Beta vulgaris* L.) stalks and leaves, grown in May and April 2016, were obtained from a producer in the city of Limeira, São Paulo state, Brazil. All reagents used for analysis were of analytical grade. High-performance liquid chromatography (HPLC)-grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ, USA), and phosphoric acid was obtained from Labsynth (Diadema, SP, Brazil). Vitexin-2-rhamnoside standard was purchased from Sigma-Aldrich (São Paulo, Brazil).

2.1. Preparation of the raw material used in the experimental diets

An overview of the preparation of the beet stalks and leaves and extract to be used for supplementation of the diets is presented in Fig. 1, Supplementary figure 1. Beet stalks and leaves were sanitized, and excess moisture was removed at room temperature. Beet stalks and leaves were divided into two groups; one group was placed on aluminium trays and taken to a domestic oven to perform the dehydration process (Clarice Brand, Pinhalzinho, SC, Brazil) at 180 °C for 45 min (SL). The samples were homogenized and stored in hermetically sealed containers at −80 °C. The other group was placed in aluminium trays, frozen and submitted to the lyophilization process (Ly) (Laboratório de Apoio Central – LAC, Faculty of Food Engineering, University of Campinas, SP, Brazil) (Supplementary figure 1). The samples were stored in hermetically sealed containers at −80 °C. The beet stalk and leaf extract that was used to supplement the mice diet (EX) was prepared only using dehydrated beet stalks and leaves (SL). Two extracts (from lyophilized and dehydrated at 180 °C/45 min) were used to determine the total and individual phenolic compound concentration in the samples. All extracts were prepared using 1.0 ± 0.1 g of oven-dried beet stalks and leaves. The raw material was placed in a conical centrifuge tube with 10 mL of ultrapure water and stirred on a tube shaker (Phoenix Luferco AP56) for 5 min, followed by centrifugation (5810 R Centrifuge, Germany) at 4000 rpm for 15 min at room temperature (25 ± 1 °C). After centrifugation, the supernatant was filtered through Whatman no. 3 filter paper, 10 mL of 80% ethanol was added to the solid residue in the conical tube, and the process was repeated under the same conditions (De Castro et al., 2019). The extracts obtained by the two sequential extractions of the same raw material were mixed, and the volume was completed to 20 mL with 80% ethanol in a volumetric flask. The extract was stored at −80 °C in amber glass until used for HPLC analysis, and the extract (EX) obtained from dehydrated beet stalks and leaves (SL) was stored at −80 °C until used to supplement the diet (EX group).

2.2. Sample preparation for the analysis of beet stalks and leaves by HPLC

Freeze-dried (Ly) and oven-dried (SL) beet stalk and leaf samples were prepared using an extraction protocol similar to De Castro et al. De Castro et al., 2019). The samples (1.0 ± 0.1 g) were extracted by two sequential extractions. The first extraction was carried out using 10 mL of water and by stirring for 5 min. After centrifugation, the supernatant was collected, and the solid residue re-extracted with 10 mL of 80% ethanol by stirring for 5 min. Both extracts were combined, and the volume brought up to 20 mL in a volumetric flask. The extracts were filtered through syringe filters (nylon, 25 mm, 0.22 μm; Analytica, Barueri, São Paulo, Brazil) before chromatographic analysis.

2.3. Identification of the compounds present in the extracts by ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC–MS/MS)

The compounds present in the extract were identified using a UHPLC–MS/MS 8040 instrument (Shimadzu, Kyoto, Japan) consisting of a liquid chromatography system coupled to a triple quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) source. Chromatographic separation was performed on a Kinetex C18 column (2.6 μm, 3.0 mm i.d., 100 mm; Phenomenex, California, USA) using a binary mobile phase. Solvent A was water, and solvent B was acidified acetonitrile (0.1% formic acid). The elution gradient used at 40 °C was as follows: 0 min, 98% A; 5 min, 98% A; 15 min, 85% A; 20 min, 80% A; 25 min, 65% A; 30 min, 20% A; 34 min, 20% A; 35 min, 98% A, at a flow rate of 0.3 mL/min. The autosampler temperature was maintained at 10 °C, and the injection volume was 10 μL. The ESI source parameters were as follows: capillary voltage, −3.5 kV; heat block temperature, 500 °C; desolvation line temperature, 250 °C; drying gas flow (N2), 10 L/min; nebulizing gas flow (N2), 1.5 L/min; collision-induced dissociation gas pressure (Ar), 224 kPa. For each compound, ESI(−)–MS/MS data were first collected for the identification of deprotonated molecules [M−H], and two of the most selective product ions were chosen for the MRM transitions using a dwell time of 20 ms. Data was acquired and processed with Labsolution software (version 5.53 SP2, Shimadzu). The recorded masses were processed throughout the chromatogram during the time interval of the peaks present at different wavelengths of detection (260, 290, 335, 360 and 484 nm). The extract was then reinjected, the detected masses were examined for fragmentation using different capillary voltages, and the detected fragments were recorded. The main compound present in the extracts was identified as vitexin-2-rhamnoside based on its molecular weight (MW 578) and the presence of the fragments (m/z) 457, 274, 413 and 293. Most compounds present in the extracts were related to vitexin, an apigenin flavone glucoside, presenting the characteristic 293 ion in the MS/MS spectra.

2.4. HPLC analysis

The HPLC analysis of the samples was carried out in an EXTRACT-US analysis system (FAPESP 2013/04304-4, patent pending), consist-

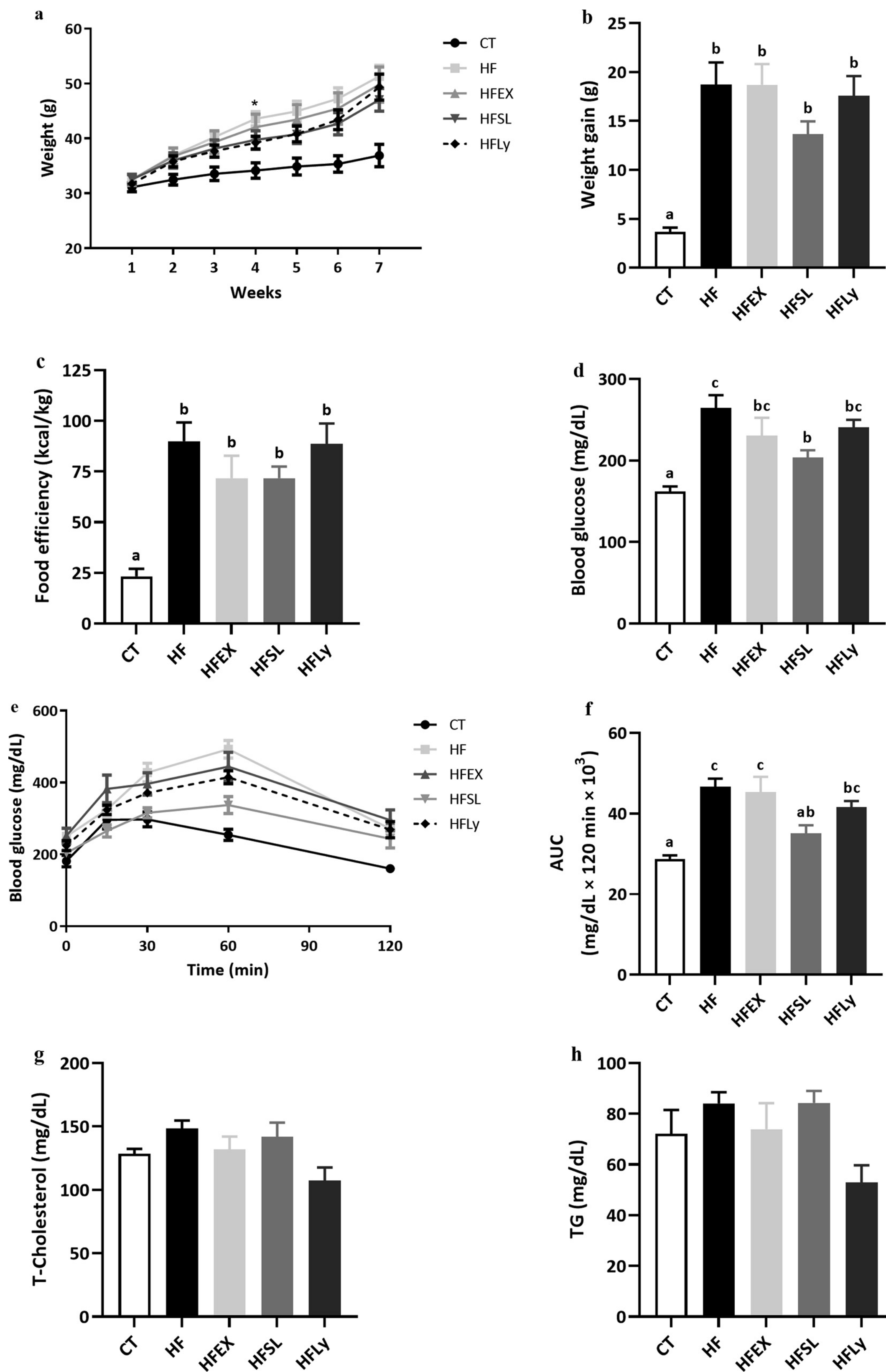

Fig. 1. Influence of supplementation with beet stalks and leaves on (A) weekly body mass gain, (B) total body mass gain, (C) food efficiency, (D) fasting glucose and glucose homeostasis assessed by (E) intraperitoneal glucose tolerance test (ipGTT) and (F) area under the curve analysis, serum (G) total cholesterol and (H) triglycerides. Control (CT); high-fat diet (HF); high-fat diet with extract of stalks and leaves (HFEX); high-fat diet with dried stalks and leaves (HFSL); and high-fat diet with lyophilized stalks and leaves (HFLy). Data are shown as mean ± SEM. One-way, LSD post hoc test, $P < 0.05$; n = 5–8 animals per group.

ing of an HPLC pump (PU2080, Jasco, Kyoto, Japan), an HPLC ternary gradient unit (LG 2080-2, Jasco, Japan), a three-line degasser (DG 2080-55, Jasco, Kyoto, Japan), a UV–Vis detector (UV-7075, Jasco) and five automatic two-position 10-port valves (Waters Corporation, Milford, MA, USA). The compounds were separated by an adaptation of the method developed by Rostagno et al. using a fused core-type column (Kinetex C18, 2.6 μm, 100 A, 100 × 4.6 mm; Phenomenex, Torrance, CA, USA) (Rostagno et al., 2011). The column was maintained at room temperature. The mobile phase consisted of water with 1% v/v phosphoric acid (solvent A), and acetonitrile with 1% v/v phosphoric acid (solvent B). The gradient profile was: 2 min, 88% A; 4 min, 80% A; 6 min, 70% A; 8 min, 40% A; 10 min, 20% A; 13 min, 20% A; and 14 min, 95% A. The equilibration time between runs was 3 min. Flow rate was 1.2 mL/min, and injection volume was 5 μL. Peaks were recorded and integrated at 320 nm. The software for the control of the system was developed by Kalatec (Campinas, SP, Brazil). ChromNav software from Jasco was used for data acquisition and processing. The vitexin-2-O-rhamnoside compound was identified by comparing the retention times of the peak obtained in analysis of the extracts to the peak obtained in analysis of the authentic standard. The vitexin-2-O-rhamnoside standard solution (100 mg/L) was diluted in a mixture of methanol and water (90:10 v/v) to prepare the calibration curve (six points: 100, 50, 25, 2.5, 1 and 0.5 ppm). The calibration curve for the compound was prepared by plotting concentration versus area. All compounds present in the samples were expressed as vitexin-2-rhamnoside equivalents (VRE). The analysis was performed in duplicate.

2.5. Experimental protocol

The experiments involving animal procedures followed the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health, and the guidelines of the Brazilian College for Animal Experimentation. Experiments were approved by the Ethics Committee on Animal Use – CEUA, UNICAMP, under protocol 4239-1. Forty Swiss male mice, 21 days old, were obtained from the UNICAMP Animal Centre. The animals were allowed to acclimate for 3 weeks before the beginning of the experiment and were then divided randomly into five groups with a similar mean weight and standard deviation: (CT) standard group (n = 8); (HF) high-fat diet group (n = 8); (HFEX) group fed high-fat diet supplemented with extract of dried beet stalks and leaves (n = 8); (HFSL) group fed high-fat diet supplemented with dried beet stalks and leaves (n = 8); and (HFLy) group fed high-fat diet supplemented with lyophilized beet stalks and leaves. Diet and water were given *ad libitum* throughout the experiment. The efficiency of diet was estimated by the equation: (weight gain/food intake [g]) × 100. The animals were kept in a temperature-controlled environment (25 ± 1 °C) with a 12-h light cycle. An analytical balance (Mark 500, Bel Engineering, Italy) was used to weigh the animals and to analyze the food intake and was checked once a week during the 8-week experiment. The experimental protocol of supplementation with beet stalks and leaves is presented in Supplemental figure 1); 0.5% of dehydrated or lyophilized beet stalks and leaves was mixed directly into the diet (HFSL and HFLy). The beet stalk and leaf extract were added directly into the diet (HFEX), and the total volume (10 mL/100 g) was adjusted to enhance the total phenolic compounds expressed as VRE/100 g diet compared to the phenolic concentration in the HFSL diet (Supplementary figure 1). Therefore, the group supplemented with dehydrated (powder) beet stalks and leaves (HFSL) received 2.04 mg VRE/100 g of diet, the animals supplemented with beet stalk and leaf extract (HFEX) received 2.04 mg VRE/100 g of diet, and the group supplemented with lyophilized beet stalks and leaves received 4.10 mg VRE/100 g of diet. All groups were treated for 8 weeks. The composition and nutritional value of the control diet and the high-fat diet are shown in Table S1 (Supplemental table S1).

2.6. Intraperitoneal glucose tolerance test (ipGTT)

Basal blood glucose levels were measured after 4 h of fasting with an Accu-Check Performa glucometer (Roche). The test was done after an intraperitoneal injection of glucose solution (1.0 g glucose/kg body weight). Blood glucose concentration was measured in blood from tail-tip bleeding and was used to determine glucose levels at 0, 15, 30, 60 and 120 min. Area under the curve values were determined by the trapezoidal method.

2.7. Serum biochemical analysis

The blood was centrifuged at 25 °C, 3,500 rpm for 30 min to separate the serum. Serum concentrations of total cholesterol and triglycerides were measured by commercial KITs (Laborlab, Brazil) of enzymatic colorimetric assay and quantified according to the manufacturer's instructions.

2.8. Haematoxylin and eosin staining

The hepatic tissues of the mice (n = 3 per group) were perfused with 4% paraformaldehyde solution during sacrifice. Then, liver sections of approximately 3 cm were fixed in 6% formaldehyde for approximately 6 h. The liver sections were then washed in running water for 40 min and processed in the following solutions: 70% alcohol solution; 80% alcohol solution; 90% alcohol solution; 99% alcohol solution; xylol I; xylol II; paraffin I; paraffin II, at 65 °C. After being embedded in paraffin, the slices were incorporated into a mould, cut into sections of 5 μm thickness using a microtome and finally stained with haematoxylin and eosin (H&E) for visualization of hepatocyte morphology. NAFLD activity score (Kleiner et al., 2005) was applied for semiquantitative analysis of the three defined criteria of NASH: steatosis (0–3), ballooning (0–3) and lobular inflammation (0–2) using a Leica DMI 4000B (Switzerland) microscope.

2.9. Western blotting

At the end of the experiment, the animals were anesthetized with a solution of sodium ketamine (0.1 g/kg), diazepam (5 mg/kg) and xylazine (3 mg/kg), and decapitation was used to cull mice that had been starved for 12 h. The negative control liver samples were weighed, frozen in liquid nitrogen and stored at −80 °C for further analysis. To evaluate insulin signalling, a bolus injection of regular insulin (5 UI) was administered (Humulin, Eli Lilly and Company, USA) through the abdominal cava vein; subsequently, hepatic samples were extracted after 45 s, this tissue being the positive control for the stimulus. The samples were homogenized in freshly prepared ice-cold buffer [1% (v/v) Triton X-100, 0.1 mol/L Tris, pH 7.4, 0.1 mol/L sodium pyrophosphate, 0.1 mol/L sodium fluoride, 0.01 mol/L EDTA, 0.01 mol/L sodium vanadate, 0.002 mol/L PMSF and 0.01 mg/mL aprotinin] using a tissue homogenizer (Bead Ruptor 12 Homogenizer, Omni International, Kennesaw, GA, USA). Insoluble material was removed by centrifugation (10,000*g*) for 30 min at 4 °C. The protein concentration of the supernatant was determined using the Biuret dye-binding method. The supernatant was suspended in Laemmli sample buffer (1 mmol sodium phosphate/L, pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% sodium dodecyl sulphate (SDS), 2% mercaptoethanol). Immunoblotting was performed by using protein extract (50 μg) from each mouse sample and polyacrylamide gels (SDS-PAGE) using a miniature gel apparatus (BioRad, Richmond, CA, USA). Electrotransfer of proteins from the gel to a nitrocellulose membrane was performed for 120 min at 120 V (constant) in a transfer buffer that contained methanol and SDS. Membranes were then blocked with a solution containing 5% fat-free milk in Tris-buffered saline (TBS)–Tween 20 (TTBS; 10 mmol Tris/L, 150 mmol NaCl/L, 0.5% Tween 20) for 2 h at room temperature. Nitrocellulose membranes

were probed overnight at 4 °C with specific antibodies [pAKT (#4060), AKT (#4691), TNFα (sc-52746), pNFκB (#3033) and β-actin (ab8227)]. The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (diluted 1:5000 in TTBS containing 3% dry fat-free milk) at room temperature, which was followed with a 2-h incubation. Bands were detected by chemiluminescence (Thermo Scientific #34078) and quantified by densitometry (UN-Scan-it Gel 6.1, Silk Scientific Inc, Orem, UT, USA).

2.10. Real-Time RT-qPCR

Total RNA was isolated from frozen hepatic tissue using TRIzol (Invitrogen, Carlsbad, California). cDNA was synthesized using 3 μg of total RNA and with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368813). PCR was run using TaqMan PCR Master Mix (Applied Biosystems). Target mRNA expression was normalized to the mean of actin and GAPD housekeeping genes and presented as a relative value using the comparative threshold cycle (Ct) method ($2-^{\Delta\Delta}$Ct).

2.11. Fatty acid profile

Hepatic total lipids were extracted from 200 mg (±1 mg) of tissue following the method of Folch (Folch, Lees, & Stanley, 1957). The samples were homogenized in 1 mL Folch solution (2:1 v/v chloroform/methanol) and centrifuged at 1,000 rpm for 10 min. Salt solution was used to increase the recovery and water was also added for phase separation in the final chloroform/methanol/water solution ratio of 8:4:3. The supernatants were collected, and the lipid-containing fraction was dried. Lipids were transmethylated as previously described (Miyamoto et al., 2018). The fatty acid profile was determined by gas chromatography, using an Agilent series 6850 GC EUA system chromatograph (Santa Clara, CA, USA). The methyl esters of the fatty acids were separated according to the AOCS Ce 1f-96 method, with the capillary column DB-23 Agilent (50%, cyanopropyl-methylpolysiloxane 60 m length, internal diameter of 0.25 mm and 0.25μmof film thickness). The following analysis conditions were employed: flow of 1.0 mL/min; linear speed of 24 cm/s; detector temperature of 280 °C; injector temperature of 250 °C; oven temperature: 110 °C for 5 min, 110–215 °C (5 °C/min), 215 °C for 24 min; carrier gas: helium, injected volume: 1.0 μL, split 1:50. The qualitative composition was determined by comparison of the peak retention times of the respective fatty acid standards and expressed in mass percentage (Table 3).

2.12. Data presentation and statistical analysis

The results are expressed as the mean ± SE of the indicated number of experiments. The Levene test for the homogeneity of variances was initially used to check the fit of data to the assumptions for parametric ANOVA. Data were analyzed by one-way ANOVA followed by LSD post hoc tests if there were significant interactions among CT, HF and HFEX; CT, HF and HFSL; or CT, HF and HFLy groups. The level of significance was set at $P < 0.05$.

3. Results

3.1. The concentration of phenolic compounds was different in the samples obtained from beet stalks and leaves

Analysis of the samples showed a complex matrix made of several different compounds at different concentrations (Table 1). The main compound present in the samples was the flavonoid vitexin-2-rhamnoside (V2R), an apigenin derivative. The UHPLC–MS/MS indicated that most compounds were associated with vitexin due to the presence of the molecular ion *m/z* 293, characteristic of this compound, and therefore were expressed as VRE.

Analysis of the samples revealed that freeze-dried and oven-dried beetroot stalks and leaves have a different chemical profile (Supplementary figure 2). Basically, the same compounds were detected but differences were observed in the amount of individual compounds, possibly caused by degradation of compounds present due to exposure to high temperatures.

V2R (peak #4) was significantly affected by the high temperature used in the oven for drying the raw material, and a much lower concentration of this compound was found in the SL sample (1.63 mg/g) as compared to the freeze-dried sample (2.93 mg/g). There was also a drastic reduction in the concentration of another vitexin derivative (peak #7), from 3.51 (Ly) to 0.74 mg VRE/g when comparing the freeze-dried sample with the oven-dried sample (SL). In contrast, the concentrations of several V2R-related compounds (peaks #5, #6 and #8) were higher in Ly when compared to SL, which can be explained by the thermal degradation of the compounds present, promoting interconversion between chemical forms.

Additionally, a lower total amount of the compounds detected by HPLC was observed in the SL sample (4.08 mg VRE/g) when compared to the HFSL sample (8.21 mg VRE/g).

The chemical profile of the extract was the same as that of the SL sample since the same raw material was used in both cases. Considering the amount of extract used to supplement the diet (10 mL), the EX group received an estimated total of 2.04 mg VRE/100 g of feed, of which 0.82 mg was V2R.

3.2. High-fat diet led to body mass gain after 8 weeks of diet

Eight weeks of high-fat (HF) diet led to increased body mass gain ($P < 0.05$) after the third week of the experiment compared to the normocaloric group (CT) (Fig. 1A), as demonstrated in the Fig. 1C, the efficiency of diet was increased in HF groups compared to CT, with no differences in the supplementation groups. There was no difference in body mass ($P > 0.05$) among HFEX, HFSL and HFLy groups (Fig. 1A and B). Regarding food intake, no significant differences were observed, with a mean consumption of: HF = 3.57 g/day (18.19 calories/day); CT = 5.25 g/day (18.92 calories/day); HFEX = 4.87 g/day (24.82 calories/day); HFSL = 3.41 g/day (17.38 calories/day) and HFLy = 3.43 g/day (17.50 calories/day), and the animals did not presented adverse symptoms as diarrhea. Serum profile showed no statistical difference in total serum cholesterol and triglycerides (TG) levels (Fig. 1G and F). These results suggest that the supplementation with beet stalks and leaves could not suppress the body mass gain of diet induced obesity (DIO) mice.

3.3. Liver histopathological characterization after supplementation with beet stalks and leaves (HFEX, HFSL and HFLy)

To further explore whether the supplementation could ameliorate hepatic steatosis induced by HF, a semi-quantitative scoring system to assess the range of histological features of NAFLD was applied. Eight weeks of HF diet feeding dramatically induced hepatic steatosis (Fig. 2B), which was evidenced by H&E staining of liver sections and NAFLD activity score (NAS) (Table 2), when compared to the control group (Fig. 2A). The group treated with EX or Ly (Fig. 2C and E) showed qualitative evidence of lower fat accumulation and ballooning compared to the HF diet group (Table 2). There was no difference between the SL supplemented group compared to the HF in the liver score of steatosis (Fig. 2D). Our results show that the HF diet was able to promote hepatic steatosis indistinctly and the score NAS evidenced only mildly ameliorated in mice treated with EX or Ly but the SL supplementation could not rescue the damage caused by lipid overload.

Table 1
Concentration of the main compounds present in the extracts.

Peak*	EX (mg/10 mL)[a]	SL (mg/g)[b]	Ly (mg/g)[c]
1	0.01	0.02	0.03
2	0.03	0.03	0.03
3	0.03	0.03	0.03
4 (Vitexin-2-rhamnoside)	0.82	0.82	1.47
5	0.04	0.04	0.09
6	0.09	0.10	0.26
7	0.37	0.37	1.76
8	0.10	0.10	0.22
9	0.04	0.04	0.04
10	0.31	0.31	0.13
11	0.03	0.03	0.03
12	0.03	0.03	0.02
13	0.15	0.15	0.04
Total	**2.04**	**2.04**	**4.10**

* Indicates the results expressed in vitexin-2-hamnoside equivalents. [a] Concentration (mg/10 mL) in the extract obtained from dehydrated beet stalks and leaves (EX); [b] Concentration (mg/g) in the dehydrated beet stalks and leaves (SL); [c] Concentration (mg/g) in the freeze-dried beet stalks and leaves (Ly).

3.4. Dehydrated powder of beet stalks and leaves ameliorated glucose homeostasis

As expected, there was an increase in fasting glucose in HF animals when compared to that of the CT group ($P < 0.05$). Interestingly, even without differences in body mass (Fig. 1 A and B) and steatosis among HF diet groups (Fig. 2), supplementation with SL (HFSL group) caused a decrease in fasting glucose levels ($P < 0.05$) (Fig. 1D). In order to analyze the glucose tolerance, a GTT was performed after 7 weeks of treatment (Fig. 1E). The HF group presented a greater area under the curve during the test when compared to the CT group ($P < 0.05$). As observed for fasting glucose, the HFSL group presented a slight reduction in the area under the curve ($P < 0.05$) (Fig. 1E and F). These results confirm that consumption of a high-fat diet increased fasting glucose and impaired glucose homeostasis in HF, HFEX and HFLy groups, but evidenced that SL treatment was able to promote a mild improvement in the glucose homeostasis.

3.5. Liver fatty acid profile after 8 weeks of supplementation with beet stalks and leaves

To further understand the connection between the results described above with the treatments we performed FA analysis to determine the quality and the quantity of FA species after the experimental period (8 weeks). No difference was observed in total fatty acid profile (SFA and MUFA) in the supplemented groups compared to the HF group. However, ARA (C20:4) content increased in the supplemented groups and were comparable to CT (Table 3). Overall, supplementation increased the total polyunsaturated fatty acid (PUFA – DHA C22:6 and ARA C20:4) levels. Further investigations are required to understand the link between beet stalks and leaves supplementation with PUFA levels alterations in the liver.

3.6. Insulin pathway and inflammatory markers assessment after 8 weeks of supplementation with beet stalks and leaves.

Dietary fat intake is one of the most important environmental factors leading to the development of insulin resistance and consequent metabolic dysfunction. After assessing metabolic parameters, we analyzed the hepatic tissue in order to determine if supplementation with beet stalks and leaves could rescue the insulin sensitivity induced by HF (Fig. 3A-F).

High-fat diet imparied glucose homeostasis in HF, HFEX and HFLy groups, however supplementation with SL (Fig. 3D-F), promoted mild

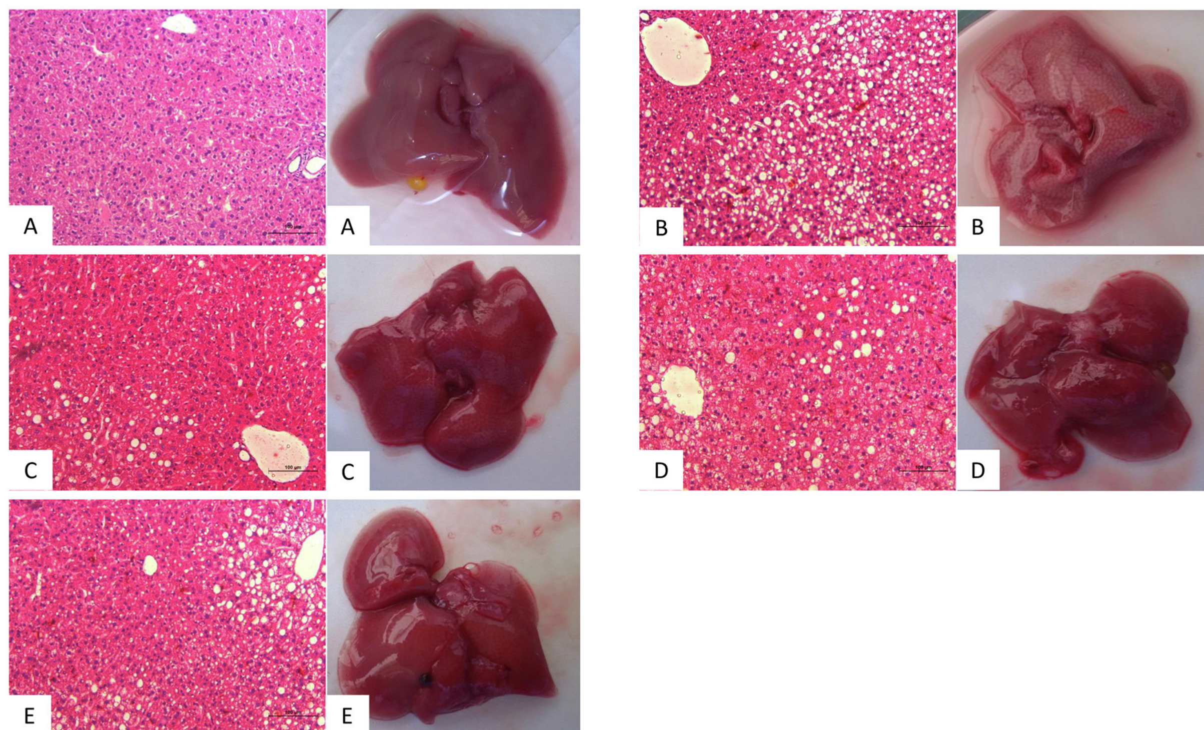

Fig. 2. Histologic grades of steatosis. Hematoxylin-eosin staining of liver paraffin sections show normal histology in (A) CT-fed and steatosis were observed in (B) HF-fed, (C) HFEX-fed, (D) HFSL-fed and (E) HFLy-fed mice. Magnification: ×20, Bar = 100 μm.

Table 2
NAFLD activity score (NAS) score in mice.

Histological features	CT	HF	HFEX	HFSL	HFLy
Steatosis	0.67 ± 0.76	1.75 ± 0.65	1.00 ± 0.00	1.50 ± 0.70	2.17 ± 0.29
Hepatocellular ballooning	0.33 ± 0.29^{b}	1.75 ± 0.65^{ab}	1.00 ± 0.00^{ab}	2.17 ± 0.29^{a}	1.25 ± 1.06^{ab}
Lobular inflammation	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are presented in mean ± SD. Different letters at the same line indicate statistical differences between experimental groups. Kruskal-Wallis, Dunn post-hoc test, $P < 0.05$.

Table 3
Fatty acid composition obtained from hepatic lipids.

Fatty acid	CT (%)	HF (%)	HFEX (%)	HFSL (%)	HFLy (%)
C 12:0 (Lauric)	0.10	0.23	0.27	0.10	0.15
C 14:0 (Miristic)	0.50	0.50	0.52	0.28	0.39
C 15:0 (Pentadecanoic)	0.08	0.10	0.10	0.09	0.10
C 16:0 (Palmitic)	23.95	22.53	21.16	21.99	22.19
C 16:1 (Palmitoleate)	3.85	1.77	1.61	1.27	1.49
C 17:0 (Margaric)	0.12	0.29	0.25	0.26	0.27
C 17:1 (Heptadecenoic acid)	0.12	0.18	0.14	0.11	0.16
C 18:0 (Stearic)	6.00	5.6	6.02	6.00	5.98
C 18:1 (Oleic)	33.32	32.81	32.40	28.88	29.10
C 18:2 (Linoleic)	18.02	25.56	25.50	27.67	26.94
C 18:3 (Linolenic)	0.53	0.60	0.55	0.60	0.60
C 20:0 (Arachidic)	0.27	0.51	0.56	0.62	0.48
C 20:1 (Gadoleic)	0.92	1.03	1.09	0.92	0.84
C 20:4 (ARA)	6.97	4.54	6.10	6.52	6.11
C 22:0 (Behenic)	0.07	0.20	0.31	0.37	0.20
C 20:5 (EPA)	0.17	0.15	0.20	0.17	0.15
C 22:5 (DPA)	0.21	0.34	0.35	0.31	0.48
C 22:6 (DHA)	4.67	2.86	2.80	3.75	4.23
C 24:0 (Lignocérico)	0.13	0.04	0.07	0.09	0.14
SFA	31.22	30.16	29.26	29.80	29.90
MUFA	38.21	35.79	35.24	31.18	31.59
PUFA	30.57	34.05	35.50	39.02	38.51
Total (%)	100.00	100.00	100.00	100.00	100.00

Standard group (CT), high-fat diet group (HF), high-fat diet supplemented with extract of stalks and leaves (HFEX), high-fat diet supplemented with dried stalks and leaves (HFSL) and high-fat diet supplemented with lyophilized stalks and leaves (HFLy).

improvements in the deleterious effect of HF and restored fasting glucose levels and glucose tolerance. We hypothesized whether HFSL improvement on glucose homeostasis could be a result of its beneficial effects on the insulin signaling in the hepatic tissue. Our data suggest that HFEX (Fig. 3A and B), HFSL (Fig. 3C and D) and HFLy (Fig. 3E and F) supplementation slightly increased activation of the insulin activity in the liver, as evaluated by AKT phosphorylation at Thr308, after insulin administration, compared to animals maintained on an HF. Next, we asked whether the alterations in the insulin signalling occur as a consequence of the low-grade inflammatory state triggered by HF. At the molecular level, we detected lower expression of *Tnfα* in CT (Fig. 3G) and decreased protein content of TNFα in CT and HFSL (Fig. 3H and Supplementary figure 3 A-C). No significant differences were observed on pNFkB (Supplementary figure 3 A-C). Overall, the results suggest that supplementation with SL decreased TNFα in the hepatic tissue, however there was no significant improvement on insulin sensitivity. Moreover, we could not discard the hypothesis that SL ameliorated fasting glucose and glucose homeostasis were due to the rescue in insulin sensitivity in extrahepatic tissues.

4. Discussion

Dietary manipulation is the basal and first-line intervention in combating obesity and obesity-associated metabolic disorders, because of access and lower cost. Beetroot (*Beta vulgaris* L.) and its non-conventional parts such as stalks and leaves are rich in phenolic compounds and may have functional properties. Previously, our results suggest that supplementation with dehydrated beet stalks and leaves may prevent some of the alterations from dietary-induced obesity in mice, decreasing fasting glucose and cholesterol levels and attenuating the oxidative stress generated by HF diet (HFD) (Lorizola et al., 2018).

In the current study, we evaluated whether supplementation with beet stalks and leaves could revert impaired metabolic parameters and ameliorate hepatic steatosis of experimental animals submitted to an HFD. We compared the effect of three types of supplementation on HFD, namely an ethanolic extract of dehydrated beet stalks and leaves (EX), dehydrated powder of beet stalks and leaves (SL) and lyophilized powder of beet stalks and leaves (Ly). In our study, we observed that animals fed an HFD for 8 weeks presented increased body mass gain and fasting glucose, glucose intolerance assessed by GTT, evidence of hepatic insulin resistance and increased inflammation compared to the normocaloric group (CT). Our findings are in agreement with the literature reporting that long-term exposure to lipid and carbohydrate overload places a heavy burden on adaptive responses that leads to continuous chronic inflammation and oxidative stress, resulting ultimately in a metabolic imbalance (Furukawa et al., 2004; Gregor & Hotamisligil, 2011; Lorizola et al., 2018; Miyamoto et al., 2018, 2020).

Regarding supplemented groups, after 8 weeks, no differences were observed in body mass and food intake (*data not shown*) among EX, SL and Ly groups. However, supplementation with SL attenuated the deleterious effect of HFD due to ameliorated glucose homeostasis and the decreased TNFα protein levels. In addition, all supplemented groups seemed to be more sensitive to insulin stimulus in the liver compared to HF-fed mice, but only the SL group presented a mild improvement on glucose homeostasis. Hepatic fatty acid profile showed increased

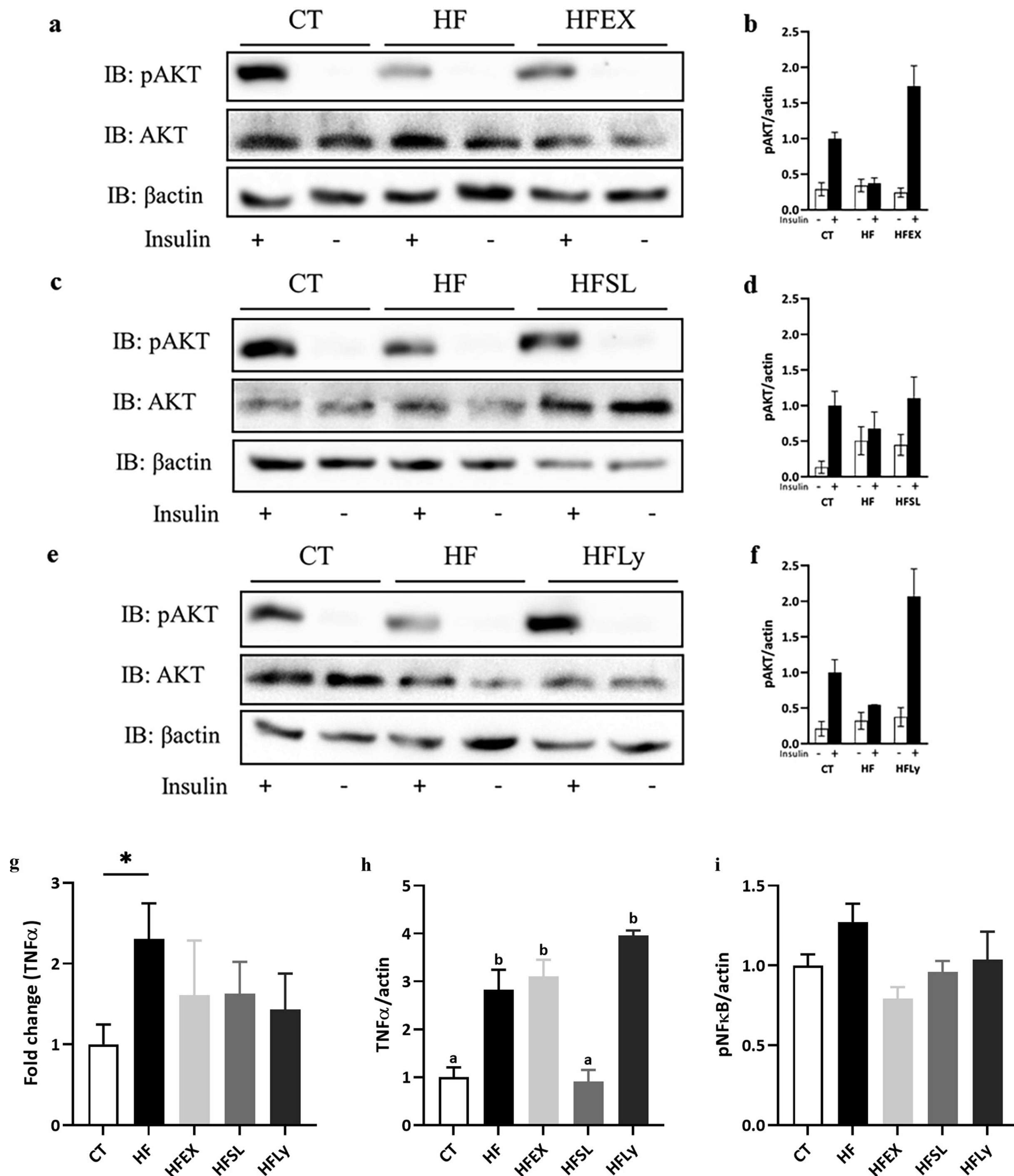

Fig. 3. Effects supplementation with beet stalks and leaves on insulin signalling and inflammatory markers. Representative Western blot analysis of protein content of pAKT in the liver after a bolus injection of saline or regular insulin (5 UI) through the abdominal cava vein. For control of gel loading, membranes were reblotted for β-actin or total proteins. The data were obtained at the end of an 8-week experimental period for Swiss mice fed on regular standard diet (CT) high-fat diet (HF) and compared with those for mice fed HF diet supplemented with (A and B) extract of stalks and leaves (HFEX), (C and D) dried stalks and leaves (HFSL) and (E and F) lyophilized stalks and leaves (HFLy). (G) TNFa gene expression assessed by RT-qPCR (n = 4 per group) and protein levels of (H) TNFa and (I) pNFkB (n = 4–6 per group). *Student's *t*-test CT × HF, $P < 0.05$. Different letters at the same graphic mean statistical differences among experimental groups. One-way, LSD post hoc test, $P < 0.05$.

levels of PUFA, such as DHA and ARA, in the EX and Ly group. Dietary PUFA are known to play a regulatory role in the lipid metabolism, as negative regulators of hepatic lipogenesis preventing hepatic steatosis (Sekiya et al., 2003; Pauter et al., 2014).

Moreover, our data indicate increased AKT phosphorylation in the liver of animals from supplemented groups, suggesting an improvement in insulin signaling. The reasons for the disconnection between liver fat /AKT signaling and the benefit for glucose homeostasis are unclear. The improvement in the glucose homeostasis found in HFSL could be a consequence of pancreatic insulin secretion and/or glucose uptake from skeletal muscle and white adipose tissues ("Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes –

2018,”). One possibility is that the HFSL group has both mechanisms occurring, which either may not have occurred to HFEX and HFLy groups or occurred in a lower intensity, leading to minor changes in glucose homeostasis.

Our results could be associated with the chemical profile of the beet stalks and leaves. Vitexin and its derivatives were the major bioactive compounds presented in the beet stalks and leaves. Vitexin (apigenin-8-C-glucoside), a flavonoid glycoside derived from apigenin, is an active component of many medicinal plants and is receiving increased attention due to its range of pharmacological effects.

Vitexin-2-O-rhamnoside-related compounds, as demonstrated in previous studies (Bumke-Vogt et al., 2014; Choo, Sulong, Man, & Wong, 2012; Gengatharan, Dykes, & Choo, 2015; He et al., 2016; Jung, Cho, & Choi, 2016; Kulabas et al., 2018; Lorizola et al., 2018; Nurdiana et al., 2017), have been shown to reduce inflammation and promote beneficial effects against many disorders caused by HFD, including the improvement of glucose homeostasis, HOMA-IR, plasma insulin and hepatic lipid metabolism, and a significant decrease in hepatic PEPCK and G6Pase activity in HFD-fed mice (Escande et al., 2013; Jung et al., 2016; Kim et al., 2014; Ono & Fujimori, 2011; Shukla & Gupta, 2010). It is important to mention that the dose of bioactive compounds, like vitexin derivatives, used in our study may be insufficient for suppressing body weight gain and body fat accumulation (*data not shown*). Further studies are needed to evaluate the dose response and duration of treatment with these bioactive compounds.

The differences in the biological effects observed between treatments (SL *vs* EX or Ly) may be related to interactions of the phenolic compounds with other components of the sample, especially proteins, present in the food matrix. According to Borgwardt et al. (Borgwardt, Bonifatius, & Gardemann, 2008), the interaction between bioactive compounds, such as genistein, and acidic peptides potentialized the inhibitory effect on platelet aggregation. The formation of genistein conjugates may improve their biological activity (Rimbach et al., 2004) and, consequently, their bioavailability during the digestion process (Borgwardt et al., 2008). In this context, the dehydration process at high temperature can promote cell disruption in the beet leaf matrix and release bioactive compounds, improving the biological activity of beet stalks and leaves. Therefore, considering our results obtained so far, it is important to investigate whether bioactive components present in the beet stalks and leaves used in our study could be involved in the regulation of hepatic gluconeogenesis and/or the insulin signaling pathway in extrahepatic tissue of mice.

In summary, our data indicate the addition of phenolic compounds in the form of dehydrated stalks and beet leaves in the HF fed animals was positive, considering some of the parameters evaluated. We observed that the form of administration, the dose used, and the time of intervention may influence the effects associated with fasting glucose and glucose tolerance. Our results suggest that there is a positive effect of the food matrix on the bioavailability of the phenolics present in dehydrated beet stalks and leaves. It is known that phenolic compounds with antioxidant potential may be in a free form and/or in a bound form in foods of plant origin. Thus, by supplementing the diet with the extract, only the free and soluble phenolic compounds are capable of being absorbed and made available to the body. Furthermore, it is necessary to carry out future studies that evaluate the dose response as a function of time. In addition, the isolation of some compounds presents in beet stalks and leaves and their administration by different means and in different experimental protocols could result in interesting data that would help to understand the possible functional properties of beet stalks and leaves. Although, some limitations of our study should be noted. Despite the treatment with EX, SL or Ly, we observed that fatty liver occurs in all four HF-fed groups. Here, we presented only qualitative evidence of hepatic triglyceride content. Thus, we were unable to assess whether improvement of glucose homeostasis in the SL-treated group is mediated by alterations on hepatic triglyceride content. However, semiquantitative analysis of NASH suggested it could not be explained by decreases in liver steatosis, since the SL group presented the same score as the HF group. Future studies would be necessary in order to clarify if the improvement in the glucose homeostasis found in HFSL could be a consequence of enhanced pancreatic insulin secretion and/or glucose uptake from skeletal muscle and white adipose tissues.

5. Conclusions

Our results suggest that beet (*Beta vulgaris* L.) stalks and leaves could be used as adjuvants in obesity to mitigate the damage generated by oxidative stress and to improve parameters related to glucose homeostasis. The dose–response relationship between the consumption of beet (*Beta vulgaris* L.) stalks and leaves and the health-related benefits remains to be elucidated.

Author contributions

I.M.L. and J.E.M.: contributed to design research, conducted research, reviewed the literature, conducted the statistical analysis and wrote the manuscript. A.L.F.V. and B.R.S.: conducted research and experimental planning. R.M.N.B., M.A.T., A.S.T., M.A.R. and M. M.: provided essential reagents, materials and financial support besides providing advice regarding interpretation of the results and editing the manuscript. M.M. and C.D.C.: were responsible for design research, provided essential reagents, materials and other financial support besides providing advice regarding interpretation of the results, conduct of the research and data collection; they also provided advice regarding the manuscript, and wrote and edited it.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2021.100018.

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